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Supplemental Information

Tks5 and SHIP2 Regulate Invadopodium Maturation, but Not Initiation, in Breast Carcinoma Cells

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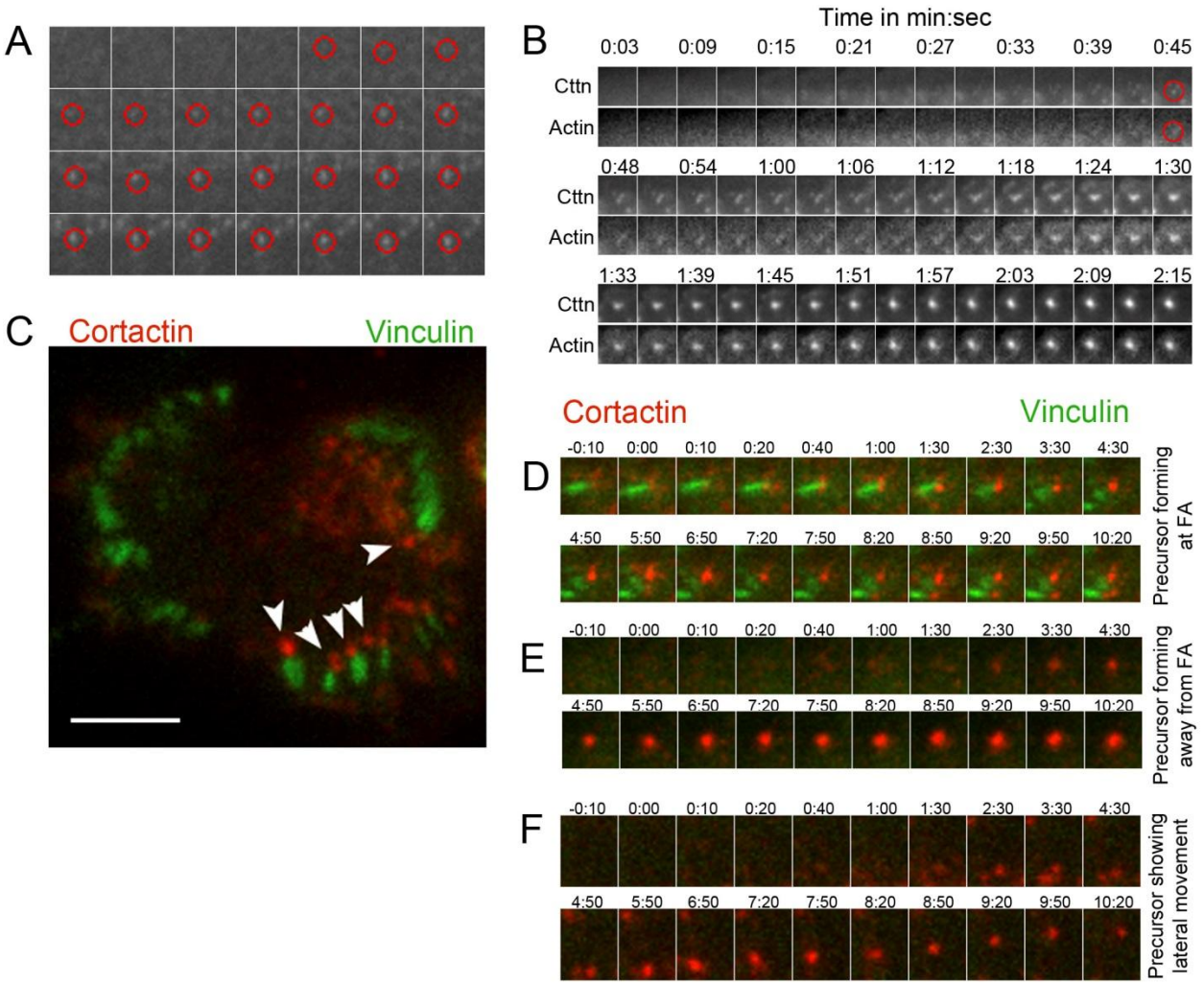

Figure S1. Actin Arrival Kinetics during Precursor Assembly and Invadopodium Precursors Initiate at the Proximal Tip of Focal Adhesions, Related to Figure 1

(A) A zoomed area of the site of an invadopodium precursor assembly from Movie S2 is shown as a time-lapse montage. Red circles outline the tracked invadopodium by "Invadopodia tracker" plugin [1]. Note that during early assembly even the faintest invadopodium precursor signal is detected by the plugin.

(B) Time-lapse montage of TagRFP-cortactin and GFP-actin fluorescence channels for a newly assembling invadopodium precursor. Red circles indicate the first appearance of cortactin and actin fluorescence puncta after EGF stimulation. Note that both cortactin and actin appear at the same time. Time 0:00 corresponds to the EGF addition.

(C) TagRFP-cortactin and GFP-vinculin cells were stimulated with 5 nM EGF and imaged with TIRF microscopy with frames every 10 sec for the first 5 min to capture the fast dynamics and then changed to frames every 30 sec for the next 10 min. Image is a still from Movie S5, which shows the location of invadopodium precursors (red) and focal adhesions (green). Note that invadopodium precursors initiate at the proximal tip of focal adhesions (white arrowheads). Scale bar = 10 μm.

(D) Time-lapse montage showing invadopodium precursor (cortactin in red) initiation occurring at the proximal tip of focal adhesion (vinculin in green).

(E) Time-lapse montage showing invadopodium precursor (cortactin in red) initiation occurring away from the focal adhesion.

(F) Time-lapse montage showing lateral motility of invadopodium precursor (cortactin in red).

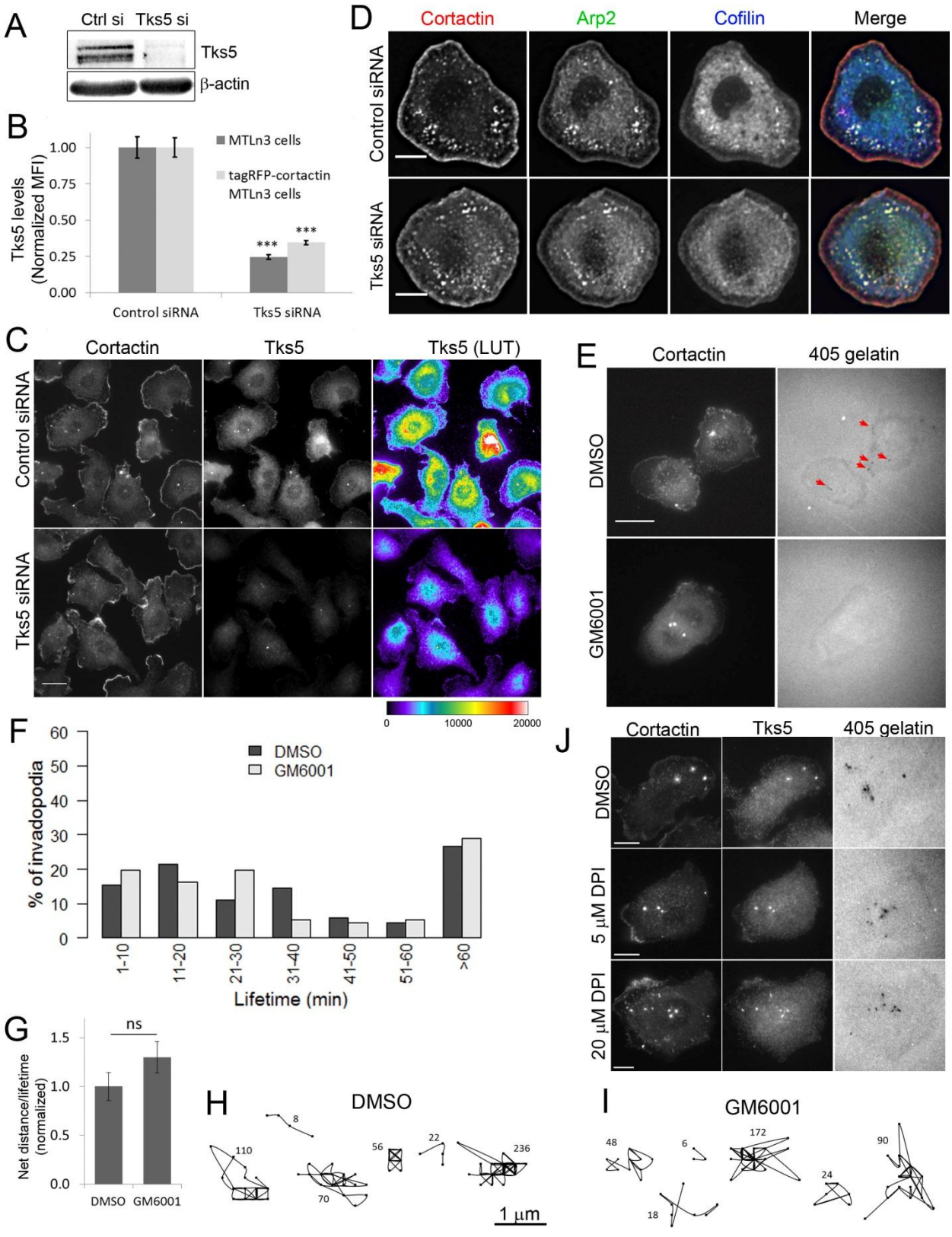
A
Ctrl si
Tks5 si
Tks5
β-actin
B
Tks5 levels (Normalized MFI)
1.00
0.75
0.50
0.25
0.00
MTLn3 cells
tagRFP-cortactin MTLn3 cells
***
***
Control siRNA
Tks5 siRNA
D
Cortactin
Arp2
Cofilin
Merge
Control siRNA
Tks5 siRNA
C
Cortactin
Tks5
Tks5 (LUT)
Control siRNA
Tks5 siRNA
0
10000
20000
E
Cortactin
405 gelatin
DMSO
GM6001
F
% of invadopodia
60
50
40
30
20
10
0
DMSO
GM6001
1-10
11-20
21-30
31-40
41-50
51-60
>60
Lifetime (min)
G
Net distance/lifetime (normalized)
1.5
1.0
0.5
0.0
ns
DMSO
GM6001
H
DMSO
8
110
70
56
22
236
1 µm
J
Cortactin
Tks5
405 gelatin
DMSO
5 µM DPI
20 µM DPI
I
GM6001
48
6
172
90
24
18

Figure S2.

Figure S2. Tks5 Knockdown and Its Effect on Invadopodium Precursor Formation, Role of Matrix Degradation in Invadopodium Motility, and Role of Reactive Oxygen Species in Invadopodia Formation and Matrix Degradation, Related to Figure 2

(A) Cells were transfected with control or Tks5 siRNA and lysates were immunoblotted with Tks5 and β-actin antibodies. Tks5 level in Tks5 siRNA sample was reduced by 85-90%.

(B) Quantification of the Tks5 mean fluorescence intensity, MFI (dark gray) in Tks5 siRNA treated cells show that Tks5 level is reduced by similar extent as with western blotting (refer to Figure S2A). To check if TagRFP-cortactin transfection affects Tks5 KD levels, stable TagRFP-cortactin MTLn3 cells were treated with either control or Tks5 siRNA and immunostained with a Tks5 antibody. Tks5 MFI (light gray) show that in these cells Tks5 level is reduced by similar extent as in parental MTLn3 cells. n=27 (control siRNA, MTLn3), n=19 (Tks5 siRNA, MTLn3), n=24 (control siRNA, tagRFP-cortactin MTLn3) and n=17 (Tks5 siRNA, tagRFP-cortactin MTLn3) cells.

(C) MTLn3 cells were treated with control or Tks5 siRNA, plated on gelatin matrix and stained with cortactin and Tks5 antibodies. Rainbow lookup table (LUT) is used to visualize the expression level of Tks5. Note that there is very low cell-to-cell Tks5 KD variability. Scale bar = 20 μm.

(D) Control or Tks5 siRNA treated cells were stimulated with 5 nM EGF for 1 min and stained with cortactin, arp2 and cofilin antibodies to identify invadopodium precursors. Note that the number of invadopodium precursors is similar in control or Tks5 siRNA cells. Scale bars = 10 μm.

(E) TagRFP-cortactin expressing cells were either treated with DMSO or 5 μM GM6001 for 16 h and time-lapse images were acquired every 2 min in the RFP channel. Red arrows show degradation spots in the 405 gelatin image for DMSO treated cell. No degradation spots were seen in GM6001 treated cells. Scale bar = 20 μm.

(F) Invadopodia lifetime histograms for DMSO and 5 μM GM6001 treated cells. Note that there is no difference in invadopodia lifetime histograms in both the cases. n=116 (DMSO) and 111 (GM6001) invadopodia.

(G) Invadopodium movement is quantified as net distance/lifetime and shows that there is no difference in invadopodium motility for DMSO and GM6001 treated cells. n=51 (DMSO) and 48 (GM6001) invadopodia.

(H and I) Representative invadopodium trajectories for DMSO and GM6001 treated cells, respectively. The number beside each trajectory is the lifetime of that invadopodium in min.

(J) Cells were treated with either DMSO or 5 μM DPI or 20 μM DPI and plated on 405 gelatin for 16 h. Cells were fixed and stained with cortactin and Tks5 antibodies to visualize invadopodia. Scale bars = 10 μm.

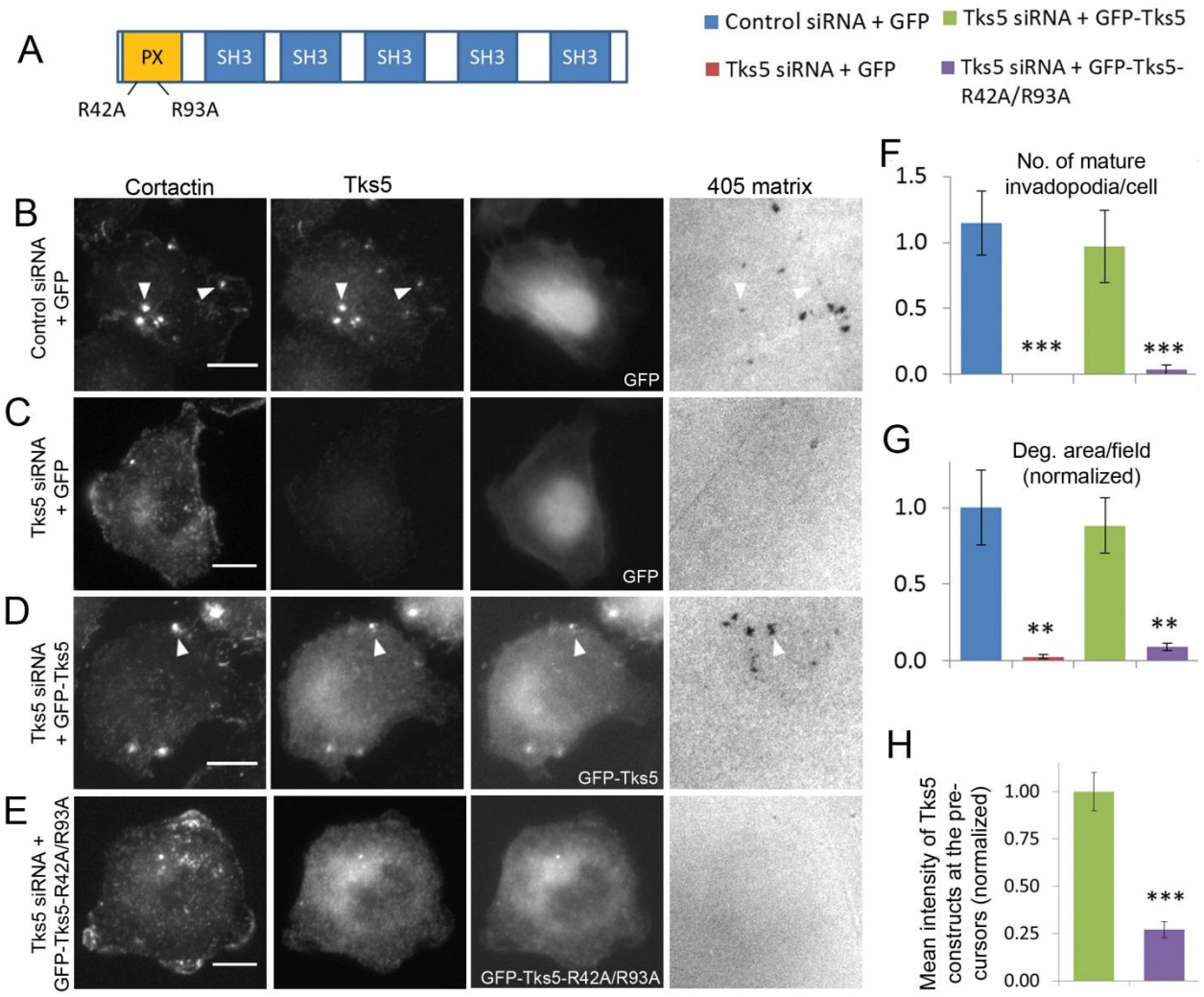

Figure S3. Effect of Tks5 KD-Rescue on Invadopodia Number and Matrix Degradation, Related to Figure 3

(A) Diagram showing different Tks5 domains and R42A, R93A mutations sites in Tks5, which disrupt its binding to PI(3,4)P_2.

(B-E) Knockdown-rescue experiments were performed where cells were treated with (B) control siRNA or (C-E) Tks5 siRNA and rescued with (B and C) GFP or (D) GFP-Tks5 or (E) GFP-Tks5-R42A/R93A constructs. Cells were plated on 405 gelatin matrix and stained with cortactin and Tks5 antibodies to identify invadopodia. White arrowheads in (B and D) point to mature invadopodia showing corresponding degradation spots in the 405 matrix channel. Scale bars = 10 μm.

(F-G) Quantification of the (F) number of mature invadopodia/cell, n=48, 43, 33 and 28 cells for the four KD-rescue conditions respectively and (G) degradation area/field of view, n=14, 14, 12 and 16 fields of view for the four KD-rescue conditions respectively.

(H) Mean fluorescence intensities of GFP-Tks5 and GFP-Tks5-R42A/R93A, in Tks5 KD cells, were quantified at invadopodium precursors. Note that there is a 75% reduction in Tks5 localization to precursors when Tks5-PI(3,4)P2 binding is disrupted. n=53 and 49 for the two conditions respectively.

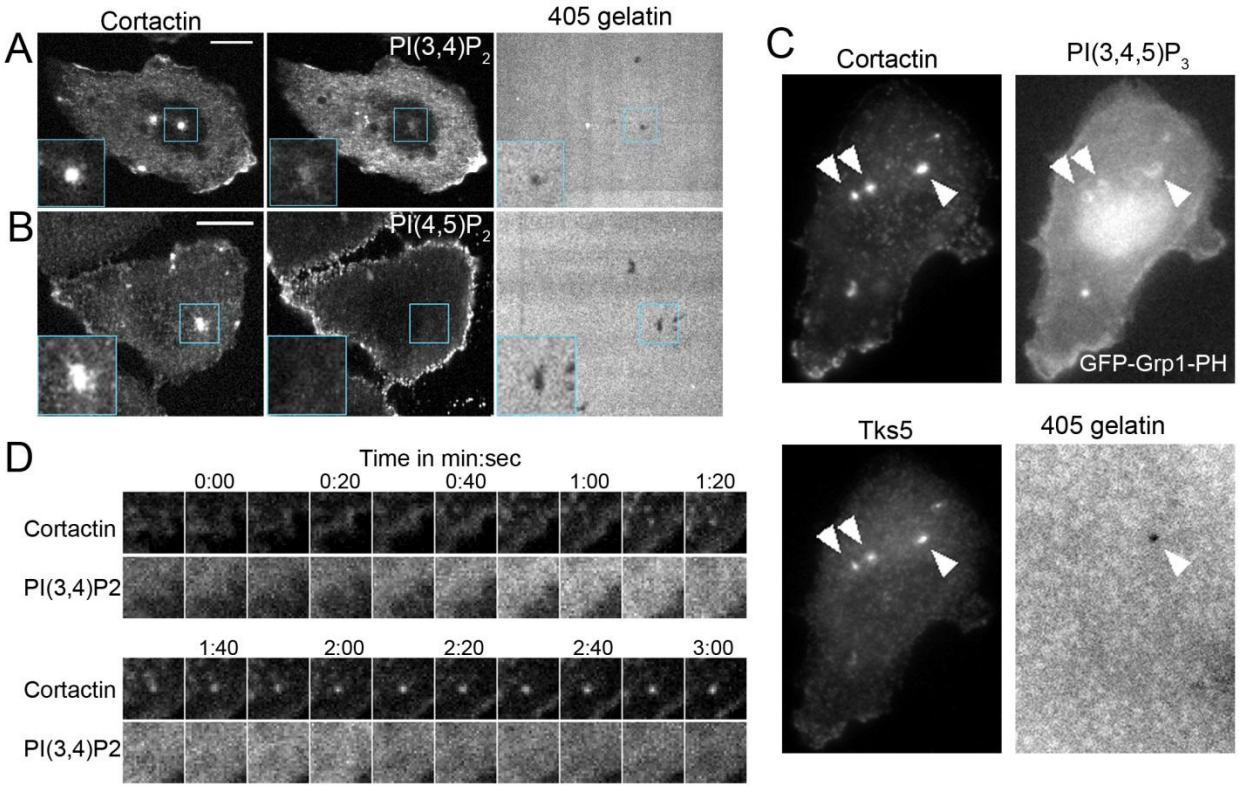

Figure S4. PI(3,4)P_2, PI(4,5)P2, and PI(3,4,5)P3 Localizations, and PI(3,4)P_2 Arrival Kinetics during Early Precursor Assembly, Related to Figure 4

(A-B) Cells were plated on 405 gelatin matrix and stained with cortactin and (A) PI(3,4)P_2 or (B) PI(4,5)P_2 specific antibodies. Zoomed insets of invadopodia and the corresponding degradation holes are also shown in the panels. Scale bars = 10 μm.

(C) Cells were transfected with GFP-Grp1-PH (specific marker for PI(3,4,5)P3), plated on 405 gelatin matrix and stained with cortactin and Tks5 antibodies to identify mature invadopodia. White arrows show ring-like patterns of PI(3,4,5)P3 accumulation around the invadopodium cores.

(D) Time-lapse montage of cortactin and PI(3,4)P_2 channels show a homogenous PI(3,4)P_2 distribution during invadopodium precursor assembly. Time 0:00 corresponds to 5 nM EGF addition.

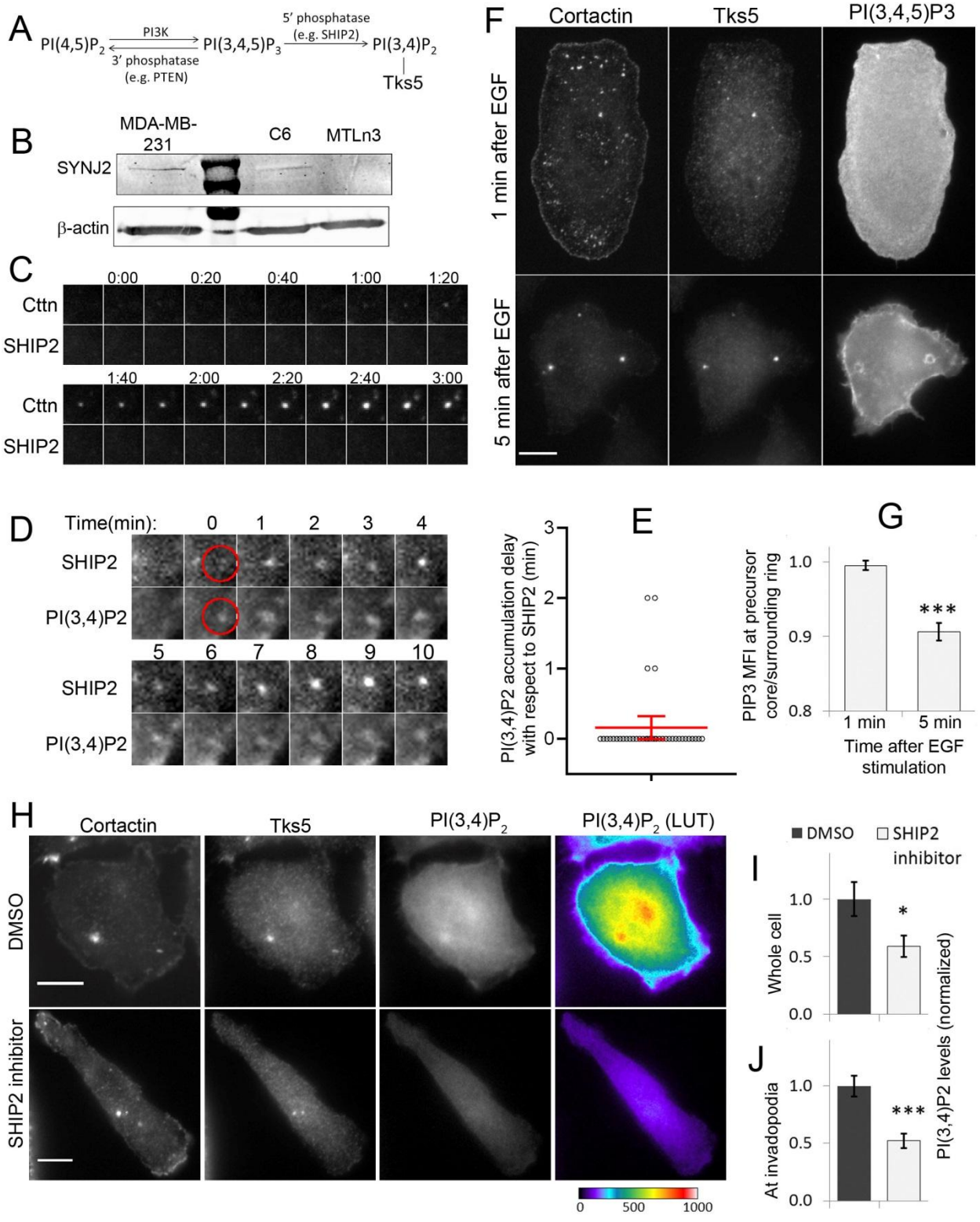
A
PI(4,5)P2
PI3K
3′ phosphatase (e.g. PTEN)
PI(3,4,5)P3
5′ phosphatase (e.g. SHIP2)
PI(3,4)P2
Tks5
B
MDA-MB-231
C6
MTLn3
SYNJ2
β-actin
C
0:00
0:20
0:40
1:00
1:20
Cttn
SHIP2
1:40
2:00
2:20
2:40
3:00
Cttn
SHIP2
D
Time(min):
0
1
2
3
4
SHIP2
PI(3,4)P2
5
6
7
8
9
10
SHIP2
PI(3,4)P2
F
Cortactin
Tks5
PI(3,4,5)P3
1 min after EGF
5 min after EGF
E
PI(3,4)P2 accumulation delay with respect to SHIP2 (min)
3
2
1
0
G
PIP3 MFI at precursor core/surrounding ring
1.0
0.9
0.8
***
1 min
5 min
Time after EGF stimulation
H
Cortactin
Tks5
PI(3,4)P2
PI(3,4)P2 (LUT)
DMSO
SHIP2 inhibitor
0
500
1000
I
DMSO
SHIP2 inhibitor
Whole cell
1.0
0.5
0.0
*
J
At invadopodia
1.0
0.5
0.0
***
PI(3,4)P2 levels (normalized)

Figure S5.

Figure S5. MTLn3 Cells Do Not Express Synaptojanin-2, SHIP2 and PI(3,4)P2 Arrival Kinetics, Changes in PI(3,4,5)P3 Levels after SHIP2 Arrival, and the Effect of SHIP2 Inhibitor on PI(3,4)P2 Levels in the Whole Cell and at the Invadopodia, Related to Figures 6 and 7

(A) Diagram showing the PI3K signaling pathway and the generation of $PI(3,4)P_2$ from $PI(3,4,5)P_3$ by the action of a 5' inositol phosphatase (e.g. SHIP2). Tks5 binding to $PI(3,4)P_2$ is shown by a solid line.

(B) MTLn3 whole cell lysate was analyzed for the synaptojanin-2 expression level by western blotting. Human MDA-MB-231 and rat C6 whole cell lysates were used as positive controls. Membranes were scanned using a high sensitivity Odyssey infrared imaging system (LI-COR) and show that synaptojanin-2 was undetected in MTLn3 cells.

(C) Time-lapse montage of cortactin and SHIP2 channels show that SHIP2 does not arrive during early invadopodium precursor assembly. Time is in min:sec. Time 0:00 corresponds to 5 nM EGF addition.

(D) Time-lapse montage from TIRF imaging of GFP-SHIP2 and TagRFP-2xTAPP1-PH transfected cells reveal simultaneous arrival of SHIP2 and PI(3,4)P2 at the invadopodium precursor. Red circles indicate the first frame of SHIP2 and PI(3,4)P2 appearance

(E) Quantification of the PI(3,4)P2 accumulation delay with respect to SHIP2. Red lines indicate mean with 95% confidence interval. n=38 invadopodium precursors.

(F) GFP-Akt-PH (PI(3,4,5)P3 marker) transfected cells were serum starved and stimulated with 5 nM EGF. Cells were fixed at 1 min and 5 min after EGF stimulation and stained with cortactin and Tks5 antibodies to identify invadopodium precursors. Note the ring-like PI(3,4,5)P3 accumulation around the invadopodium precursor core 5 min after EGF (i.e. after SHIP2 arrival) and homogenous PI(3,4,5)P3 at the invadopodium precursor core 1 min after EGF (i.e. before SHIP2 arrival).

(G) GFP-Akt-PH mean fluorescence intensity was quantified at the precursor core/surrounding ring 1 min (i.e. before SHIP2 arrival) and 5 min (i.e. after SHIP2 arrval) after EGF stimulation. n=23 (1 min), n=22(5 min) invadopodium precursors

(H) GFP-TAPP1-PH (PI(3,4,)P2 marker) transfected cells were treated with DMSO or 10 μM SHIP2 inhibitor, AS1949490 and stained with cortactin and Tks5 antibodies to identify invadopodia. Rainbow lookup table (LUT) shows that $PI(3,4)P_2$ levels are down, both at the whole cell level and at the invadopodia, in SHIP2 inhibitor treated cells. Scale bars = 10 μm.

(I-J) Quantification of (I) whole cell and (J) invadopodial $PI(3,4)P_2$ levels in DMSO and 10 μM SHIP2 inhibitor treated cells. (I) n=22 (DMSO), 20 (SHIP2 inhibitor) cells. (J) n=56 (DMSO), 52 (SHIP2 inhibitor) invadopodia.

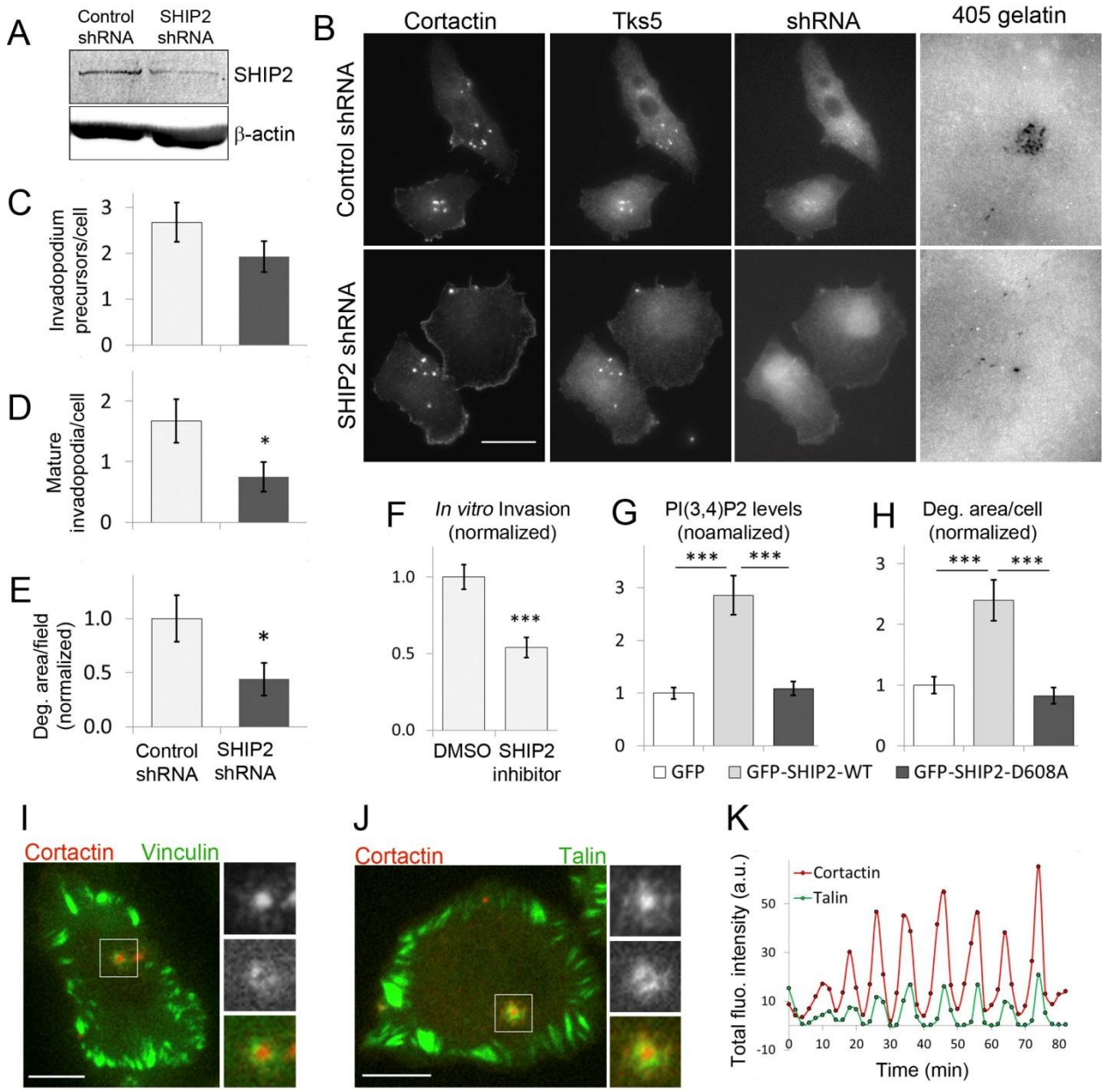

Figure S6. SHIP2 Regulates Invadopodia Maturation, Degradation Area, In Vitro Invasion, and PI(3,4)P2 Levels, and Invadopodia Are Dynamic Structures that Show Oscillations in the Cortactin Core and the Surrounding Focal Adhesion Ring, Related to Figure 7

(A) Cells were transfected with luciferase (control) shRNA or SHIP2 shRNA, whole cell lysates were prepared after 48 h and immunoblotted with SHIP2 and β-actin antibodies. SHIP2 level in SHIP2 shRNA sample was reduced by 60%.

(B) Cells were transfected with luciferase (control) shRNA or SHIP2 shRNA, plated on 405-labeled gelatin, fixed and stained with cortactin and Tks5 antibodies. GFP fluorescence in the shRNA constructs was used to identify cells with relatively similar shRNA expression level. Scale bar = 20 μm.

(C-E) Quantification of the number of invadopodium precursors per cell, number of mature invadopodia per cell and degradation area per field of view, respectively. (C-D) n=40 (control sh), n=40 (SHIP2 sh) cells (E) n=26 (control sh), n=24 (SHIP2 sh) fields of view

(F) A quantification of the effect of SHIP2 inhibition on cell invasion, n=22 (DMSO and SHIP2 inhibitor each) fields of view.

(G) Cells were transfected with TagRFP-2xTAPP1-PH as a marker for PI(3,4)P2 and GFP or GFP-SHIP2-WT or GFP-SHIP2-D608A, phosphatase inactive mutant. Whole cell PI(3,4)P2 levels were quantified. n=69, 40 and 55 cells respectively

(H) Cells were transfected with GFP or GFP-SHIP2-WT or GFP-SHIP2-D608A and plated on 405 gelatin matrix for 16 h, imaged and quantified for degradation area per cell. n=82, 69 and 69 cells respectively

(I) MTLn3 cells were transfected with TagRFP-cortactin and GFP-Vinculin and plated on gelatin matrix. Time-lapse imaging was performed on a TIRF microscope in GFP and RFP channels with frames every 2 min. Image is a still from a movie with zoomed inset showing invadopodium core with a surrounding vinculin ring.

(J) MTLn3 cells were transfected with TagRFP-cortactin, infected with CellLight Talin-GFP (Life Technologies) and plated on gelatin matrix. Time-lapse imaging was performed on an epi-fluorescence microscope (Delta Vision) in GFP and RFP channels with frames every 2 min. Image is a still from Movie S8 with zoomed inset showing invadopodium core with a surrounding talin ring.

(K) Total fluorescence intensities for the cortactin core and talin ring plotted with respect to time show oscillations in both the invadopodium core and the surrounding focal adhesion ring. Time period of these oscillations is approximately 10 min. Scale bars = 10 μm.

Supplemental Experimental Procedures

Cell Culture

Rat mammary adenocarcinoma, MTLn3 cells were cultured in α-MEM media, supplemented with 5% FBS and antibiotics as described earlier [2]. In all EGF stimulation experiments, cells were serum starved in 0.35% BSA in L-15 media for 3-4 h and then stimulated with 5 nM EGF. MDA-MB-231 were cultured as described [3], C6 rat glioma cells were from ATCC and cultured as recommended by the manufacturer, hematopoietic RBL-2H3 cells were from Dr. Jon Backer (Albert Einstein College of Medicine) and cultured as described [4].

Antibodies, DNA Constructs, and Transfection

Tks5 (sc-30122) and Arp2 (sc-15389) antibodies were from Santa Cruz Biotechnology. Cortactin (ab-33333) antibody was from Abcam. SHIP2(#2730) and SHIP1 (#2728) antibodies were from Cell Signaling Technology. N-WASP(AE922) and cofilin (AE774) antibodies were custom-made in the lab [5]. PI(3,4)P_2 (D143-3) antibody was from MBL International, PI(4,5)P_2 antibody, clone AM-212 was from Masato Umeda (Kyoto University, Japan) [6, 7], synaptojanin 2 antibody was from Marc Symons (Feinstein Institute for Medical Research, NY) and β-actin (AC-15) antibody was from Sigma-Aldrich. GFP-Tks5 was from Sara Courtneidge (Sanford-Burnham Institute, La Jolla, CA), GFP-TAPP1-PH was from Christina Mitchell (Monash University, Australia), Venus-2xHrs-FYVE and TagRFP-2xTAPP1-PH were from Dr. Tsukasa Oikawa (Keio University, Tokyo, Japan) and pcDNA3-Akt-PH-GFP was from Addgene (plasmid #18836). GFP-Tks5-R42A/R93A mutant was made by site-directed mutagenesis (Stratagene). By comparing this mutant's expression in cells with endogenous Tks5 staining, we confirmed that Tks5 mutant does not degrade *in vivo* faster than endogenous Tks5 suggesting that it folds properly (Figure S3E).

Cells were transfected with Lipofectamine 2000 (Life Technologies) using manufacturer's protocol and left to express the plasmid(s) for 24 h before imaging.

RNAi

AllStars Neg. Control siRNA (1027281) was from Qiagen, rat Tks5 siRNA (5'-CGUUAGAGGUGUCCGAAGA-3') was from Dharmacon (Thermo Scientific). MTLn3 cells were transfected with siRNA using Oligofectamine (Life Technologies) for 48 h, as described previously [8]. Luciferase shRNA and rat SHIP2 shRNA (5'-GGATTAGCATTGATAAGGA-3') were cloned in the Gertler lab.

Live-Cell Imaging for Protein Arrival Kinetics

Cells were transfected with TagRFP-cortactin and either GFP-Tks5 or YFP-N-WASP or GFP-cofilin or GFP-actin for 6-8 h, trypsinized and plated on gelatin matrix overnight. Cells were serum starved for 3-4 h before stimulating with 5 nM EGF. Cells were imaged, one frame every 3 sec, on a custom-build epi-fluorescence microscope [1], which was equipped with a CRIFF (Continuous Reflective-Interface Feedback Focus System, Applied Scientific Instrumentation) unit for maintaining the focus during the course of imaging. Microscope was also equipped with a high performance cooled 12 bit CCD SensiCam^{QE} camera (Cooke Corp.), a 60x 1.42 NA objective lens and a 6R/94T mirror as a dichroic to accommodate multi-channel imaging.

To objectively identify the frames when fluorescence (Tks5, cortactin etc.) signal first appear, we tracked newly forming invadopodium precursors using a custom written ImageJ plugin - "Invadopodia tracker" [1]. The plugin accurately tracks and identifies the first frame of fluorescence signal appearance at the site of invadopodium precursor assembly (Figure S1A, Movie S2).

Live-Cell TIRF Imaging for Arrival Kinetics Experiment

For PI(3,4)P2 accumulation and SHIP2 arrival studies with respect to cortactin, cells were transfected with TagRFP-cortactin and either GFP-TAPP1-PH or GFP-SHIP2. Cells were either serum starved, stimulated with 5 nM EGF and imaged with frames every 10 sec; or imaged in L-15 + 5% FBS media at steady state with frames every 1 min. For SHIP2 – PI(3,4)P2 arrival kinetics, cells were transfected with GFP-SHIP2 and TagRFP-2xTAPP1-PH and imaged in L-15 + 5% FBS media at steady state with frames every 1 min.

Long Time-Lapse Live-Cell Imaging for Invadopodia Stability
Stable TagRFP-cortactin expressing MTLn3 cells were transfected with control siRNA or Tks5 siRNA and plated on gelatin matrix for 16 h. Cell media was changed to L-15 + 5% FBS and imaged on a Delta Vision microscope (Applied Precision) equipped with a CoolSNAP HQ^2 CCD camera and a 60x, NA 1.42 objective lens. Cells were imaged at 37^0C, one frame every 2 min for up to 10 h.

Live-Cell Imaging of Tks5 KD-Rescue Cells
Cells were treated with either control or Tks5 siRNA and rescued with either GFP or WT Tks5 or mutant Tks5 (R42A/R93A). Cells were cotransfected with TagRFP-cortactin as a maker for invadopodia. Cells were stimulated with 5 nM EGF and imaged on Delta Vision microscope (Applied Precision). Imaging was performed at 37^0C, one frame every 10 sec for the first 4 min, followed by one frame every 30 sec for the next 2 min, followed by one frame every 2 min for the next 24 min, for a total of 30 min of imaging.

Invadopodium Degradation Assay
Alexa Fluor 405-labeled gelatin was prepared as described [1]. Cells were plated on labeled gelatin matrix overnight, fixed and stained with appropriate antibodies and imaged on a Delta Vision epi-fluorescence microscope (Applied Precision Inc.), equipped with a CoolSNAP HQ^2 camera and a 60x, NA 1.42 objective lens. Invadopodium precursors were identified as cortactin and Tks5 positive puncta without the degradation hole. Mature invadopodia were identified as cortactin and Tks5 positive puncta colocalizing with degradation hole.

Immunofluorescence
For cortactin, Tks5, N-WASP and SHIP2 antibodies, cells were fixed in 3.7% paraformaldehyde for 20 min and permeabilized in 0.1% Triton-X-100 for 5 min. For cofilin, Arp2, $PI(3,4)P_2$ and $PI(4,5)P_2$ antibodies, cells were fixed as described [2]. In both methods, cells were blocked in 1% BSA + 1% FBS for 1 h, and incubated in primary and secondary antibodies for 1 h each. Secondary antibodies (Alexa 488, 555, 647, Goat anti-mouse, -rabbit, -chicken) were from Life technologies.

Western Blotting
Cell lysates were prepared by washing cells in cold PBS and adding SDS-PAGE sample buffer. Cells were transferred to an eppendorf tube, sonicated and heated at 95 °C for 5 min. Samples were loaded in polyacrylamide gels, ran for 1.5 h, transferred to nitrocellulose membranes, blocked in Odyssey blocking buffer (LI-COR Biosciences), incubated with primary antibodies overnight at 4 °C. After 3 washes in TBS-T, membranes were incubated with secondary antibodies (Mouse 680 and Rabbit 800 [LI-COR Biosciences]) for 1 hr at RT, washed three times in TBS-T and scanned using a high sensitivity Odyssey Infrared Imaging System (LI-COR Biosciences). β-actin was used as a loading control.

Invadopodia Trajectory and Lifetime Analysis
Invadopodia lifetimes and x-y trajectories were calculated with a custom written ImageJ plugin, "Invadopodia Tracker" [1]. Briefly, 16-bit time-lapse stacks were opened in ImageJ and the invadopodia-of-interest were selected with multi-point selection tool. For linking the same invadopodium from one frame to the next, the maximum allowable invadopodium displacement was chosen to be 3-5 pixels. Invadopodia trajectories were plotted in Excel and lifetime histograms were made in R using custom-written scripts.

Inhibitor Experiments
Following inhibitors were used: SHIP2 inhibitor, AS1949490 (3718) from Tocris Bioscience at 10 μM; GM6001 from Enzo Life Sciences at 5 μM; Diphenyleneiodonium Chloride (DPI) from Calbiochem at 5 μM and 20 μM. Cells were treated with inhibitor for 16 h.

Transwell Invasion Assay
Invasion assay was performed as described before [3]. Briefly, bottom surface of 8.0 μm control inserts (BD BioCoat) were coated with 10 μg/ml fibronectin and left for 1 h in the 37 °C incubator. Excess fibronectin was removed and 250 μl reduced growth factor Matrigel (final concentration, 2.5 μg/ml) was added to the upper chamber of inserts and 750 μl, α-MEM was added to the lower chamber and left for 1 h in the 37 °C incubator. Excess Matrigel was removed from both chambers and they were allowed to

equilibrate in α-MEM at 37 °C for 1 h. After equilibration, bottom chamber was filled with 750 µl complete media (5% FBS/α-MEM). 50K cells were resuspended in 250 µl, 0.5% FBS/DMEM and plated in the upper chamber. DMSO or 10 µM SHIP2 inhibitor was added to both chambers. Cells were allowed to invade for 16 h followed by fixation in 4% PFA for 20 min and 0.2% crystal violet staining for 15 min at RT. Cells that did not invade were removed from the upper surface of the inserts using cotton swabs. Random fields on each insert were imaged on Delta Vision microscope with a 20X objective.

Statistical Analysis

Unless otherwise specified in the figure legends, statistical significance was calculated using unpaired, two-tailed Student's *t* test and statistical significance was defined as P value < 0.05. The * indicates P value <0.05; ** indicates P value <0.01; *** indicates P value <0.001 and error bars represent the SEM. For each experiment, ≥ 2 independent replicates were performed.

Supplemental References

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